I. Overview

The following progress report covers the third quarter (7/1/93-9/30/93) for contract number N00014-93-C-0019. Four topics are included in the contract; each topic's goal and current statuses are as follows:

1. The production of anti-ferret IgA antibodies. Status: **Mice immunized with ferret sIgA. Preparing sufficient sIgA for immunization of rabbits.**

2. The purification of lipopolysaccharide from *Shigella* and *Campylobacter* species. Status: **Culturing and extracting LPS of two *Shigella* species.**

3. The development of an enzyme immunoassay for the detection of enteroaggregative *Escherichia coli* heat-stable toxin. Status: **Work suspended until new EAST1 recombinants received.**

4. The production of monoclonal antibodies against strain-specific antigenic epitopes on *Campylobacter coli* flagella. Status: **Mice immunized with flagellin in preparation for hybridoma production.**

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II. Current Progress

1. Production of Anti-ferret IgA Antibodies

Purification of sIgA. During the past quarter, we continued to modify our purification methodology (presented in Second Quarter Report) to improve our yields of ferret sIgA. During the second quarter of the project, we determined that the zwitterionic detergent N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate (DDAPS) at a 1% (w/v) concentration effectively dissociates protein aggregates that are present in the ferret milk. We later discovered that 1% (w/v) DDAPS interferes with the binding of ferret sIgA to DEAE sepharose, thus reducing the yield. Previous experimentation had indicated that lower concentrations of DDAPS do not effectively dissociate the protein aggregates; thus, it was not possible to improve binding by using a lower DDAPS concentration throughout the purification procedure. In addition, we discovered that reduction of the DDAPS concentration alone does not result in complete binding of the ferret sIgA to the DEAE; therefore, we adjusted the salt content, the pH, and the detergent concentration of the DEAE sample buffer. In the previous protocol, the DDAPS-dissociated material was loaded onto the DEAE column in 4.3mM phosphate buffer, pH 7.2, containing 137mM NaCl and 2.7mM KCl. We

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now dilute the DDAPS-treated material in 10mM Tris buffer (TB), pH 8.5, containing 100mM NaCl, then load the sample onto the DEAE column. Using the modified conditions, we have improved the binding efficiency of the sIgA to the DEAE to approximately 100%.

Secretory IgA from rabbit colostrum has been reported to elute from DEAE at NaCl concentrations of 0.2M and 0.3M (Cebra and Robbins, 1966). We have produced and analyzed similar fractions. One fraction (eluted with 300mM NaCl) contains sIgA with very low levels of contaminants. The other fraction (eluted with 150mM NaCl) contains sIgA and a 50kD contaminant. Based upon silver stained gels, we have estimated that each sIgA fraction contains approximately half of the total sIgA that is bound to the column; therefore, we experimented with various elution strategies in order to separate the sIgA from the 50kD contaminant. Despite alterations in the pH and salt concentration, the contaminant continued to co-elute with the sIgA.

Examination of the clarified ferret milk by SDS-PAGE and silver stain revealed the 50kD contaminant to be a major component of the milk. The contaminant was present in the sIgA fraction obtained by size-exclusion chromatography in Sephacryl S-400, suggesting that it is either aggregated to the sIgA or is a degradation product of sIgA. Under denaturing conditions, the allotypic variant IgA2m(1) exists as heavy chain and light chain dimers (Loomes et al, 1991). Although DDAPS is a non-denaturing detergent, it is possible that the 50kD contaminant is a light chain dimer released by the DDAPS treatment. To resolve this question, we performed western blot analysis of the contaminant using polyclonal α-ferret IgG (α-H & L, Kirkegaard & Perry) as a probe. The α-ferret IgG possesses antibodies against both isotype-specific and isotype-common epitopes; therefore, milk components of immunoglobulin origin should react with the α-ferret IgG. The 50kD material did not react with the α-ferret IgG. Thus, it is unlikely that the contaminant is a light chain dimer or is of immunoglobulin origin.

Immunization of Mice. Since alteration of the elution scheme for the DEAE column did not remove the 50kD contaminant, we chose to determine its immunogenicity rather than further modify the purification protocol. If the contaminant is poorly antigenic, its presence in the inoculum would have little consequence, and so it would be unnecessary to devote additional time to the development of a protocol for its removal.

For the immunization study, two female BALB/c mice were immunized with ferret sIgA. One mouse (M1) was immunized with the 300mM NaCl elution product which contained highly purified sIgA,

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while the other mouse (M2) received the 150mM NaCl elution product which contained sIgA and the 50kD contaminant. Following each boost, serum was collected and tested by Western blot against clarified ferret milk to determine the specificity of the IgG response. Examination of sera taken prior to immunization demonstrated that neither mouse possessed endogenous antibodies which reacted with ferret sIgA when tested at a 1:50 dilution.

Following the second boost, the sera from both mice possessed antibody titers in excess of 1:4000 and were strongly reactive with the sIgA. The serum from M1 had little cross-reactivity to ferret IgG but did react with other milk components. Cross adsorption of M1 serum with ferret IgG removed all reactivity with the exception of that against the sIgA and a milk component which appeared to migrate at the dye front. This material may be J-chain (MW=14-20kD). No immunoreactivity against IgM in the ferret milk was seen. Since IgM pentamers contain J-chain, this observation suggests that if α-J-chain antibodies are present, they react with epitopes which are inaccessible when the molecule is associated with IgM. Therefore, the presence of these α-J-chain antibodies will not reduce the specificity of the cross-adsorbed sera. The serum from M2 reacted strongly with the IgG as well as sIgA. Additionally, reactivity against the 50kD protein could now be seen. This reactivity remained even after all IgG reactivity was removed by adsorption, thus reconfirming that the contaminant is not immunoglobulin related.

We are currently continuing the immunization of the mice. The results to this point indicate that the purified sIgA is a good immunogen and that isotype-specific antibodies predominate as the immunization schedule progresses. In addition, the antigenicity of the 50kD contaminant necessitates the use of only the 300mM NaCl elution product for the immunization of animals.

Purification of sIgA for Immunization of Rabbits. In addition to the mouse study, we are purifying sIgA for the immunization of rabbits. For the purification, we replaced the initial 12,000x*g* centrifugation for clarification of the ferret milk with a 100,000x*g* ultracentrifugation. Ultracentrifugation of ferret milk improves the separation of the milk components by size-exclusion chromatography as well as prevents the production of back-pressure on the column (Second Quarter Progress Report). Unexpectedly, a larger casein pellet was produced by the subsequent acid precipitation, and we could no longer separate the sIgA from the IgG by size-exclusion chromatography on Sephacryl S-400. We believe that the improved clarification resulting from ultra-centrifugation reduced the hydrophobicity of the solution, thus allowing improved precipitation of the casein which exists as

micelles (McKenzie, 1967). These micelles may have been responsible for the existence of protein aggregates in our initial clarified ferret milk preparation. Since our purification protocol was developed to accommodate the existence of large aggregates, we modified our procedure to compensate for the reduction or removal of these structures.

In our current version of the sIgA purification protocol, IgG is removed from the clarified ferret milk by affinity chromatography on a Staphylococcus Protein A Sepharose column prior to size-exclusion chromatography on Sephacryl S-300. By removing the IgG, we are now able to adequately separate the sIgA from the other milk components. All other portions of the procedure have remained unchanged with the exceptions of the use of Sephacryl S-300 instead of S-400 and the use of a Centriprep-30 for the second ultrafiltration step which is now performed prior to the addition of DDAPS to the sample. The modified method yields ~40μg of sIgA per ml of milk. The sIgA preparations have very low levels of contaminants. Due to the low yield, we will alter the rabbit immunization protocol by reducing the inocula to 250μg for the primary injection and 125μg for all subsequent boosts. Based upon the strong antibody responses of the immunized mice, we believe that the smaller inocula should not adversely affect the production of α-ferret sIgA antibodies.

2. Extraction and Purification of Lipopolysaccharide

Extraction of *Shigella* LPS. Small-scale experimentation with *S. sonnei* 53LB has shown that we can achieve a LPS yield of ~3% of the starting dry cell weight by the Westphal and Jann (1965) phenol-water extraction method. Based upon this result, we have determined that approximately 25 grams of partially dried cell mass (~5 grams of pellet per 2 liters of culture) is necessary for the production of the 200mg of LPS which was specified in the proposal. At present, we have collected the required amount of *S. sonnei* 53LB cell mass and will extract the LPS in the near future. In addition, we have recently received *Shigella flexneri* strain 2457, serotype 2A from Dr. P. Guerry of NMRI and are currently cultivating and collecting cells. Should similar LPS yields be achieved, we will need to collect the same dry cell weight.

Extraction of *Campylobacter jejuni* LPS. Cultivation and collection of *Campylobacter jejuni* 81176 cells has produced a yield of cell mass that is much lower than that of *S. sonnei* 53LB. At present, we recover ~1 gram of cell mass per 1.6 liters of broth culture overlay. In addition, a preliminary extraction using the phenol-water method of Westphal and Jann (1965) produced a yield of less than 0.05%. This is not surprising since the campylobacter contain

rough-type (R-type) LPS which is more hydrophobic than the smooth-type (S-type) LPS for which the phenol-water method works well. A method for the extraction of campylobacter LPS has been reported by Naess and Hofstad (1984). The method involves the treatment of campylobacter cells with pronase prior to extraction of the LPS by the phenol-water method. The pronase treatment removes a glycoprotein microcapsule which inhibits efficient extraction of the LPS (Conrad and Galanos, 1990). We will experiment with this method next. If pronase treatment does not significantly improve our yields, we will then try the method of Galanos et al (1969) which was developed for the extraction of R-type LPS from *Salmonella* species.

3. Enteroaggregative *Escherichia coli* Heat-Stable Toxin 1 (EAST1)

Purification of EAST. Dr. Stephen Savarino of the Naval Medical Research Institute has kindly provided us with two recombinant strains of *E.coli*. The first recombinant, TB1pSS121, possesses the EAST1 gene, *astA*, in a pMALp2 vector (New England Biolabs, Inc.). When induced with isopropyl β-D-thiogalactoside (IPTG), TB1pSS121 expresses a periplasmic fusion protein composed of EAST1 and maltose-binding protein (MBP). The ligation of MBP to EAST1 facilitates the purification of the fusion protein from other periplasmic components by affinity purification on an amylose column. Additionally, the inclusion of a factor Xa cleavage site between MBP and EAST1 provides a means for the separation of the two components. Passage of the cleavage products through an amylose column will remove the MBP, and pure EAST1 will be recovered in the flow-through.

The second recombinant, HMS174(DE3)pSS122, contains the *astA* gene in a pET11c vector (Novagen). When induced with IPTG, the transformant secretes active, recombinant EAST1 as demonstrated by Ussing chamber experiments (Savarino et al, 1993).

We have completed our examinations of both recombinants and have not been able to establish the presence of EAST1 either as a fusion protein or as a free molecule. For the recombinant TB1pSS121, we examined whole cell lysates and periplasmic material by SDS-PAGE. We were able to visualize the MBP, but we could not determine the presence of a fusion protein. Proteins isolated by affinity chromatography of periplasmic material from both the recombinant and a control containing the pMALp2 plasmid with no insert did not reveal any definitive differences between the sizes of the purified products. Additionally, treatment of affinity purified periplasmic material from TB1pSS121 with Factor Xa did not release any detectable low molecular weight molecules as determined by SDS-PAGE on a 10-20% gradient gel.

Examination of the culture filtrate from the other recombinant, HMS174(DE3)pSS122, revealed the presence of a small molecular weight molecule (~2kD) whose production appeared to be increased by induction with IPTG. The culture filtrate of a negative control containing the pET11c plasmid without an insert exhibited the same low molecular weight protein, however. No other potential EAST1 bands were found. In addition, examination of the two culture filtrates by reverse-phase high pressure liquid chromatography did not reveal any significant differences between the recombinant and the negative control.

4. Type-Specific Campylobacter Flagellin Epitopes

Production of α-Flagellin Monoclonal Antibodies. In the second quarter of the contract, we developed a whole cell ELISA for screening the hybridomas as well as screening the mice prior to immunization. During the past quarter, we examined the sera of female BALB/c mice from two sources. Mice from Simonsens (Gilroy, CA) possessed endogenous IgM which was strongly reactive with whole cells of both strains of *C. coli*. No IgG nor IgA reactivity was detected. When tested for reactivity to purified T2 flagellin, one of the three mice possessed reactive IgM. Whereas this percentage of mice containing flagellin-reactive antibody was less than that reported (100%) by Dr. T.J.G. Raybould in his second progress report for the Campylobacter Phase I project, it is not inconsistent with his finding since each screening involved only three mice. For this reason, three mice were purchased from the National Cancer Institute (NCI), and their sera were tested in the whole cell ELISA. The three mice possessed IgM which reacted with whole cells of *C. coli* VC167T2, but none possessed antibody (IgM, IgG, or IgA) which reacted with purified flagellin. It is evident that the presence of endogenous IgM which is reactive with T1 and T2 whole cells will be present regardless of the source of the animals. As we detected anti-flagellin antibodies in one Simonsens mouse, combined with the fact that purified flagellin will be used as an immunogen, we have decided to use the NCI mice.

Immunization of mice. Following the first boost, all three mice possessed IgG titers that were >1:4000 in the whole-cell ELISA. The responses to whole cells of *C. coli* VC167T1 were as much as two-fold greater than those against strain VC167T2 from which the purified flagellin was extracted. We believe that this is due to different levels of expression of common flagellar epitopes, thus producing similar results as those reported for the reactivity of LIO 8 typing sera with purified flagellin from VC167T1 and VC167T2 (Harris et al, 1987). We have attempted to remove all cross-reactive antibodies by cross-adsorption of the sera with VC167T1 cells in order to demonstrate the presence of antibodies against

strain-specific epitopes, but we have been unsuccessful so far. Cross adsorption does, however, reduce the response against VC167T1 cells to a greater extent than the response against VC167T2 cells, indicating the presence of antibodies against strain-specific epitopes.

III. Plans for Present Quarter

1. Production of Anti-Ferret IgA Antibodies

We intend to continue the immunization of mice with ferret sIgA and will attempt to induce ascites production in order to obtain a positive control for analysis of the rabbit sera. If good ascites production is induced, we will provide some to the Navy Researchers if they so desire. We will also complete production of enough purified sIgA for the immunization of the rabbits and begin the immunization regimen.

2. Extraction and Purification of Lipopolysaccharide

We will continue to culture and collect cells for the extraction of LPS. We hope to complete the purification of the LPS from the two *Shigella* species by the end of this quarter. Due to the low cell mass yields and the as yet unknown LPS yields, we do not anticipate collecting enough *C. jejuni* 81176 cells for the production of the specified 200mg during the coming quarter.

3. EAST1

Following a conversation with Dr. Savarino, it was decided that he would create new recombinants for use in the isolation of the toxin; therefore, we have temporarily suspended work upon this portion of the project. Due to the current delay, the extent of the proposed work that will be completed for this portion may be reduced due to time limitations of the contract.

4. Type-Specific Campylobacter Flagellin Epitopes

We intend to produce and screen hybridoma cells during November. If mAbs against strain specific epitopes are not found, we will attempt a second fusion. If the desired mAbs are found, we will proceed with the cloning and characterization of the hybridomas.

IV. References

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